Biochem. J. (1983) **213**, 305–311
Printed in Great Britain

Long-chain betulaprenol-type polyprenols from the leaves of *Ginkgo biloba*

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(*Received 4 January 1983/Accepted 30 March 1983*)

A long-chain betulaprenol-type polyprenol mixture was isolated from the leaves of *Ginkgo biloba* mainly as acetate. The structure was determined by mass spectroscopy, ^{1}H-n.m.r. spectroscopy and ^{13}C-n.m.r. spectroscopy. The mixture contained polyprenols-14–22, predominantly polyprenols-17, -18 and -19, and consisted of the dimethylallyl terminal unit (ω-terminal), two *trans*-isoprene residues, a sequence of 11–19 *cis*-isoprene residues and a terminal hydroxylated isoprene unit (α-terminal) aligned in that order. The concentration of these polyprenols in leaves increased from 0.04 to 2.0% of dry wt. with maturing of the leaves, though the content of total lipids was constant. The distribution of chain length in these polyprenols showed little variation throughout the whole life of the leaves.

Much attention has been directed in recent years towards the nature of dolichols and their derivatives as sugar carriers in the biosynthesis of glycoprotein (Waechter & Lennarz, 1976; Hemming, 1977). Dolichols are a mixture of α-saturated polyprenol homologues consisting of two *trans*-isoprene residues and different numbers (m) of *cis*-isoprene residues. The alignment of the *trans*- and *cis*-isoprene residues was determined as shown in formula (I) (Y. Tanaka & A. Kageyu, unpublished work).

Dolichols of different chain lengths have been isolated from animals (Dallner & Hemming, 1981), plants (Brett & Leloir, 1977) and micro-organisms (Richards & Hemming, 1972; Jung & Tanner, 1973). However, the content of dolichols in the tissues is so small (less than 0.03% of wet wt.), except for human internal organs (0.01–0.3% of wet wt.) (Rupar & Carroll, 1978), that it is difficult to obtain a sufficient amount of dolichols for comprehensive investigations.

Several types of polyprenols have been found in the plant kingdom during the past three decades. Most of the polyprenols isolated from the leaf tissues of angiosperms have consisted of three internal *trans*-isoprene units and all other residues including α-terminal unit in the *cis*-configuration. The alignment of the *cis*- and *trans*-isoprene residues in ficaprenol-11 isolated from *Ficus elastica* was determined by ^{13}C-n.m.r. spectroscopy to be as shown in formula (II) ($m = 7$) (Tanaka & Takagi, 1979).

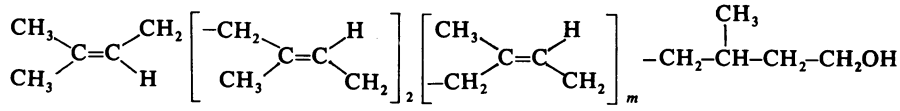
In contrast with the ficaprenols, only two examples containing two *trans*-isoprene residues (III) are recognized in polyprenols: betulaprenols-6–9 ($m =$ 3–6) from the woody tissue of *Betula verrucosa* (Wellburn & Hemming, 1966*b*), and bacterial polyprenol-11 ($m = 8$) from *Lactobacillus plantarum* (Gough *et al.*, 1970). The content of these polyprenols is fairly small (0.004–0.03% of wet wt.). Although the chain lengths of these polyprenols are shorter than those of mammalian dolichols, the fundamental alignment of the *trans*- and *cis*-isoprene residues is expected to be identical with that of dolichols, except for the absence of the saturation in the α-terminal residue.

We decided to undertake a series of investigations to search for more abundant sources of polyprenols of betulaprenol-type with $m > 10$ as starting materials for the synthesis of mammalian dolichols. The present paper reports the isolation and the structural characterization of such polyprenols from the leaves of *Ginkgo biloba*. Polyprenols consisting of two *trans*-isoprene residues and 11–19 *cis*-isoprene residues were obtained in a high yield, up to 2% of dry wt. of leaves. These polyprenols were found to have the same alignment of the *trans*- and *cis*-isoprene residues as that of mammalian dolichols.

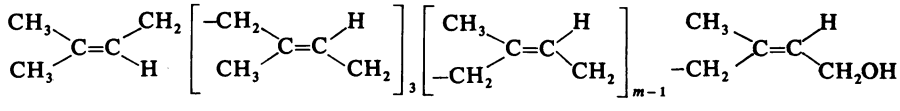
Methods

Isolation and purification of polyprenols

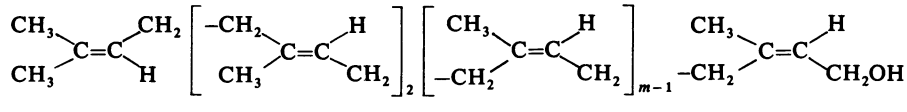
The leaves were collected from the same *Ginkgo biloba* tree (about 30 years old, male) seven times at



(I)



(II)



(III)

intervals of about 1 month after bud unfolding until leaf shedding. These leaf samples (each 200 g) were dried for 1–2 days at 50–60°C in an air-circulating drying oven. The dried leaves (each 50 g) containing 5–9% moisture were crushed into pieces of about 5 mm diameter and extracted three times with 600 ml of solvent for 3–9 days for each extraction at room temperature (approx. 20°C). The most effective extraction was performed with acetone/n-hexane (1:1, v/v). Lipids (3.814 g) containing 0.900 g of polyprenyl acetates (1.97% of dry wt. of leaves) were obtained from the leaves harvested in November.

The lipid extract was subjected to column chromatography on silica gel NW-6201 (Yamani Chemicals, Osaka, Japan) packed into a 30 mm-diameter column. Elution with n-hexane/diethyl ether (19:1, v/v) gave 1.243 g of polyprenyl acetate fraction (purity 72%), which had R_F 0.41 on t.l.c. on silica gel 60 F_{254} plates (Merck, thickness 0.25 mm) developed with n-hexane/ethyl acetate (19:1, v/v). Elution with n-hexane/diethyl ether (17:3, v/v) gave 0.251 g of polyprenol fraction (purity 7%), which had R_F 0.44 on t.l.c. on the same silica gel 60 F_{254} plate developed with n-hexane/ethyl acetate (17:3, v/v). Then 0.934 g of polyprenyl acetate fraction (purity 96.4%) was obtained by gel-permeation chromatography on a preparative column (21.2 mm internal diam. × 600 mm) packed with high-resolution styrene/divinylbenzene gel (Tanaka *et al.*, 1982*a*), with chloroform as eluent. The isolated pale-yellow oily substance was dissolved into about a 20-fold volume of acetone, and 0.015 g of waxy precipitate was filtered off. The 0.900 g amount of pure polyprenyl-14–22 acetates (and a trace amount of polyprenyl-23 acetate) was further fractionated into each component by using a reversed-phase high-pressure liquid-chromatography column (10 mm internal diam. × 300 mm) packed with Nucleosil $5C_{18}$ (Macherey–Nagel) with acetone/methanol (9:1, v/v) as eluent at a flow rate of 3 ml/min monitored by a Knauer 98.00 differential refractometer.

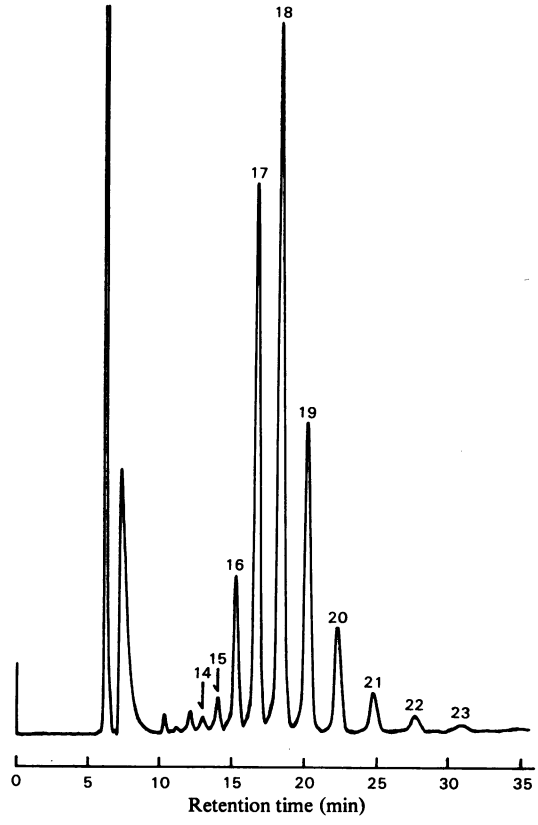


Fig. 1. *Reversed-phase high-pressure liquid chromatography of polyprenyl acetates*
For experimental details see the text.

Measurements

Mass spectra were determined in the field-desorption mode with a JEOL JMS D-300 gas chromatograph–mass spectrometer. Perfluoropoly-(propylene oxide) was used as a reference compound for the calibration of mass numbers. ^{1}H-n.m.r. and ^{13}C-n.m.r. spectra were obtained with JEOL FX-200 and GX-400 spectrometers at 200 MHz and 400 MHz for ^{1}H n.m.r. and 50.1 MHz and 100.5 MHz for ^{13}C n.m.r. Measurements were made in C^2HCl_3, with tetramethylsilane as an internal standard, at room temperature. I.r. spectra were obtained with a Digilab FTS-200 C/D Fourier-transform infrared spectrometer. Measurements were made for the samples in KBr discs at room temperature.

Results and discussion

Distribution of chain length

Fig. 1 shows the distribution of chain length in the polyprenyl acetate mixture determined by reversed-phase high-pressure liquid chromatography. The field-desorption mass spectrum of the same mixture showed a similar distribution of molecular ions corresponding to the distribution of chain length as shown in Fig. 2. The mass numbers (m/z) are reasonably assigned to the molecular ions of polyprenyl-14–22 acetates; theoretical values are 1012, 1080, 1148, 1216, 1284, 1352, 1420, 1488 and 1556 respectively. Saponification products from the polyprenyl acetate mixture gave a field-desorption mass spectrum with peaks at 970, 1038, 1106, 1174, 1242, 1310, 1378, 1446 and 1514, which are in accord with the theoretical values for polyprenols-14–22. Therefore the peaks in Fig. 1 are reasonably assigned to polyprenyl-14–22 acetates (and a small peak due to polyprenyl-23 acetate), which were confirmed by field-desorption mass-spectral measurements of the isolated polyprenyl acetates and the polyprenols obtained by saponification thereof. The percentages by weight of polyprenyl-14–23 acetates were found to be 0.9, 1.6, 6.5, 24.9, 36.5, 17.7, 6.7, 2.9, 1.6 and 0.8%, the average number of isoprene units being 17.9, from the peak areas in Fig. 1. Here, it was checked that the weight fractions of polyprenyl acetates were directly proportional to the peak areas of high-pressure liquid chromatograms, with a maximum

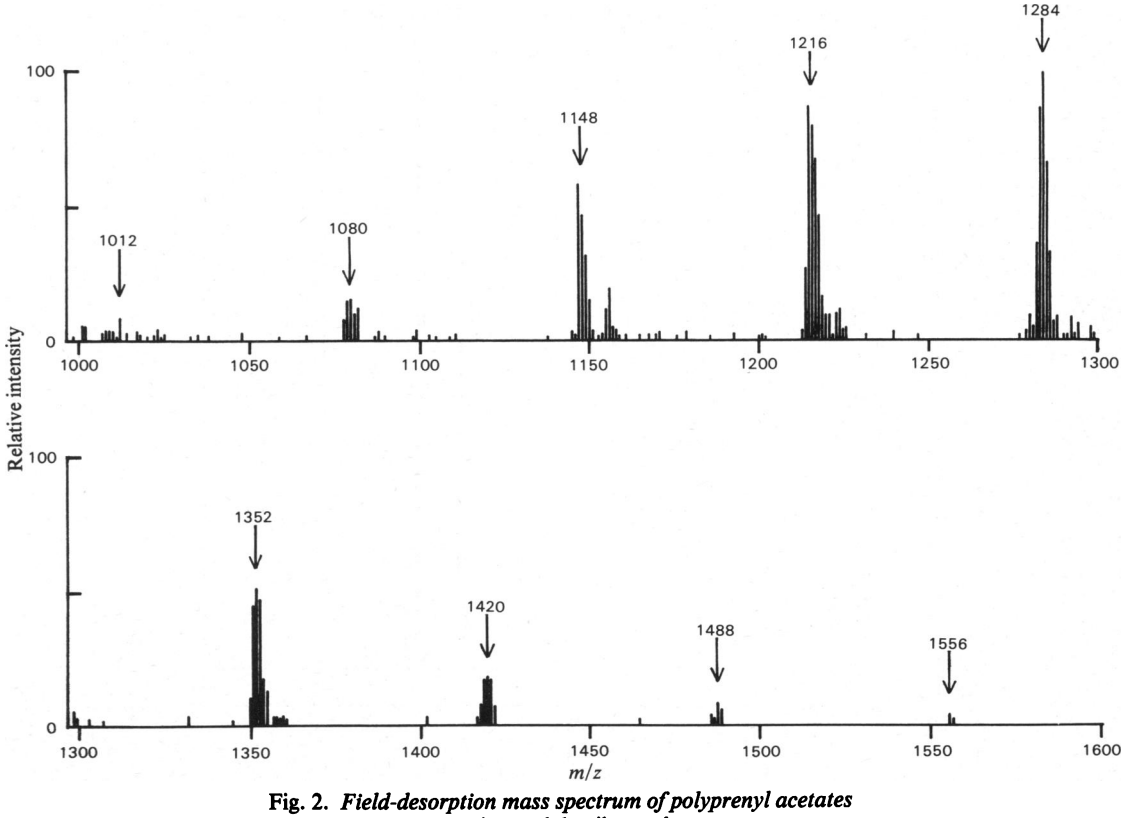


Fig. 2. *Field-desorption mass spectrum of polyprenyl acetates*
For experimental details see the text.

deviation from linearity of ±3%. These findings indicate that the polyprenyl acetates from the leaves of *Ginkgo biloba* are a mixture of homologues with a distribution of chain length similar to that of mammalian dolichols.

Structural characterization

Polyprenyl-18 acetate isolated from the polyprenyl acetate mixture showed i.r.-absorption bands characteristic of *cis*-isoprene residues and acetate, which is in good agreement with previously reported spectra (Burgos *et al.*, 1963; Wellburn *et al.*, 1967). The other polyprenyl acetates gave essentially the same spectra.

Fig. 3 shows the ^{1}H-n.m.r. spectrum of polyprenol-18 obtained by saponification of polyprenyl-18 acetate. The spectrum is in agreement with those of *cis–trans*-polyprenols (Feeney & Hemming, 1967; Stone *et al.*, 1967; Wellburn *et al.*, 1967); the signals at 1.68 and 1.60 p.p.m. are assigned to the methyl protons of the internal *cis*- and *trans*-isoprene residues respectively. Both signals also include those due to the methyl protons of the ω-terminal unit in the *cis*- and *trans*-configurations. A small signal at 1.74 p.p.m. is assigned to the methyl protons of the α-terminal *cis*-isoprene unit. The other polyprenols showed the same signals, indicating that the α-terminal *cis*-isoprene unit is a common structure for these polyprenols. The observed relative intensities are in good agreement with calculated values determined on the assumption of the presence of two internal *trans*-isoprene residues for each polyprenol, as listed in Table 1. These findings demonstrate that the polyprenols are a series of homologues consisting of two internal *trans*-isoprene residues and different numbers of internal *cis*-isoprene residues.

The alignment of the internal *cis*- and *trans*-isoprene residues can be determined by the ^{13}C-n.m.r. method (Tanaka *et al.*, 1982*b*). Polyprenol-18 showed the same ^{13}C-n.m.r. signals as ficaprenol-11 (Tanaka & Takagi, 1979), as shown in Fig. 4(*a*). The signals are assigned by the comparison of the chemical shifts with those of model compounds (Tanaka *et al.*, 1982*b*), and also by the application of INEPT (insensitive nuclei enhanced by polarization transfer) ^{13}C-n.m.r. measurement (Doddrell & Pegg, 1980), as listed in Table 2. The $C_{(1)}$-methylene carbon atoms exhibited three signals around 32–40 p.p.m., reflecting the linkage of the *cis*-

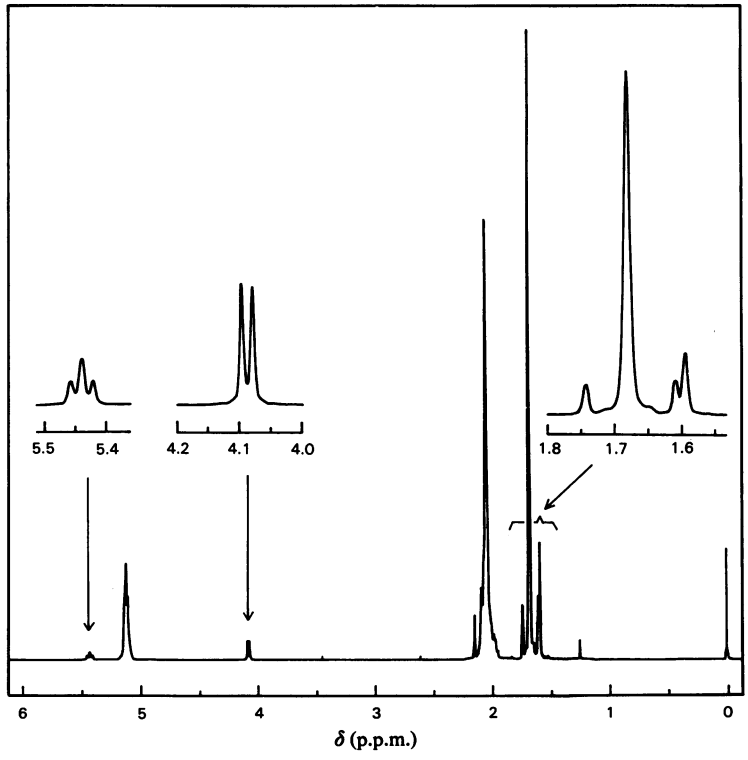


Fig. 3. ^{1}*H-n.m.r. spectrum of polyprenol-18*
For experimental details see the text.

Table 1. *Relative intensities of ^{1}H-n.m.r. signals in polyprenols-15–20*
Theoretical values are in parentheses. For experimental details see the text.

Chemical shift (p.p.m.)	Assignment	Number of isoprene units 15	16	17	18	19	20
1.60	CH_3 *trans*	3.06*	3.04*	2.93*	3.09*	2.97*	2.98*
1.61	CH_3 *trans*(ω)	(3)	(3)	(3)	(3)	(3)	(3)
1.68	CH_3 *cis,cis*(ω)	11.9*	13.0*	14.1*	14.9*	16.1*	17.0*
		(12)	(13)	(14)	(15)	(16)	(17)
1.74	CH_3 *cis*(α)	1.08*	0.93*	0.97*	0.98*	0.96*	1.03*
		(1)	(1)	(1)	(1)	(1)	(1)
4.08, 4.10	CH_2OH	1.84	1.97	1.96	1.92	1.96	2.05
		(2)	(2)	(2)	(2)	(2)	(2)
5.12	=C*H*	14.0	15.0	16.0	17.0	18.0	18.9
		(14)	(15)	(16)	(17)	(18)	(19)
5.42, 5.44, 5.46	=C*H*–CH_2OH	1.12	1.03	1.07	1.06	1.01	1.00
		(1)	(1)	(1)	(1)	(1)	(1)

* The observed and theoretical values for the methyl protons are the number of methyl groups.

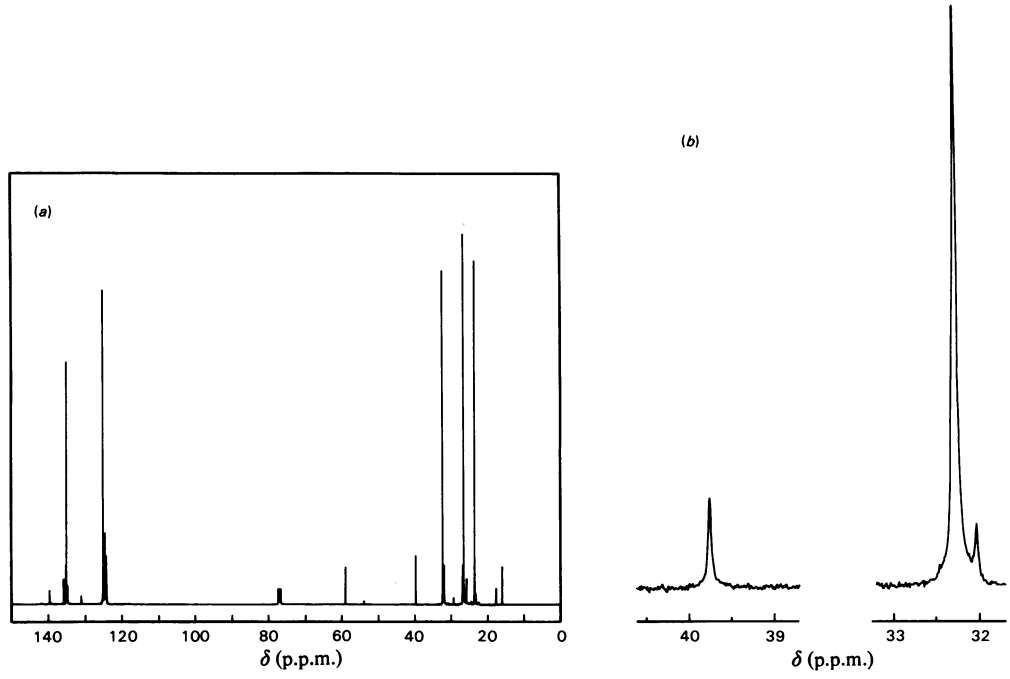


Fig. 4. *^{13}C-n.m.r. spectrum of polyprenol-18 (a) and expanded spectrum of $C_{(1)}$-methylene carbon signals (b)*
For experimental details see the text.

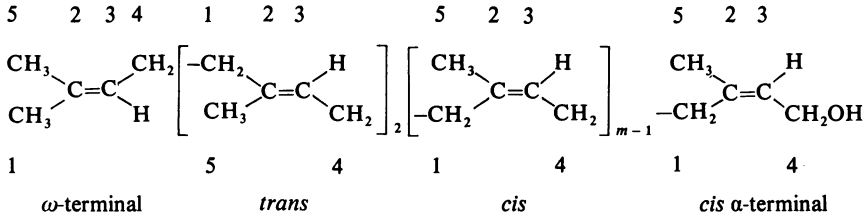
and *trans*-isoprene residues, as shown in Fig. 4(*b*), where the carbon atoms are designated as follows:

$$-C_{(1)}-C_{(2)}(C_{(5)})=C_{(3)}-C_{(4)}-$$

The signal at 39.78 p.p.m. is assigned to the $C_{(1)}$-methylene carbon atoms of the *trans*-isoprene residue in the *trans–**trans***- and *ω–**trans***-linkages. The signals at 32.29 and 32.05 p.p.m. are assigned to the $C_{(1)}$-methylene carbon atoms of the *cis*-isoprene residue in *cis–**cis***- and *trans–**cis***-linkages respec-

Table 2. *Assignment of* ^{13}*C-n.m.r. signals in polyprenols-15–20*

For experimental details see the text. The carbon atoms are designated as follows:



Chemical shift (p.p.m.)	Assignment	Chemical shift (p.p.m.)	Assignment
15.99	5-*trans*	124.28	3-ω, 3-α
17.65	5-ω	124.32	
23.42	5-*cis*, 5-α	124.52	3-*trans*
25.65	1-ω	124.62	
26.49	4-*cis*	124.75	
26.71	4-*trans*	124.98	3-*cis*
26.87	4-ω	125.12	
32.05	1-*trans*–***cis***	131.04	2-ω–***trans***
32.29	1-*cis*–***cis***	134.85	2-***trans***–*trans*
39.78	1-*trans*–***trans***	135.15	2-*cis*
	1-ω–***trans***	135.27	
58.99	4-α	135.31	
		135.96	2-***trans***–*cis*
		139.56	2-α

Table 3. *Relative intensities of* ^{13}*C-n.m.r. signals in polyprenols-17–19*

For experimental details see the text. Integrated relative intensities were obtained by gated decoupling ^{13}C-n.m.r. measurement. Theoretical values are in parentheses.

Chemical shift (p.p.m.)	Assignment	Number of isoprene units 17	18	19
32.05	*trans*–***cis***	0.96 (1)	0.92 (1)	0.93 (1)
32.29	*cis*–***cis***	13.0 (13)	14.1(14)	15.1 (15)
39.78	*trans*–***trans***, ω–***trans***	2.09 (2)	2.00 (2)	2.03 (2)

tively (Tanaka *et al.*, 1982*b*). The absence of the signal around 40.0p.p.m., which is characteristic of the *cis*–***trans***-linkage, indicates that the *trans*-isoprene units are incorporated in the ω–*trans*–*trans*-linkage. The presence of the ω–*trans*-linkage is also confirmed by the characteristic $C_{(2)}$-carbon signal of the ω-terminal unit at 131.04p.p.m.; the ω–*trans*-linkage in model compounds showed a signal around 131.0–131.3p.p.m., and the ω–*cis*-linkage a signal around 131.5–131.6p.p.m. (Tanaka *et al.*, 1983). The relative intensities of the signals reflecting the *trans*–***trans***- + ω–***trans***-, *cis*–***cis***- and *trans*–***cis***-linkages were determined for polyprenols-17, 18 and 19, as listed in Table 3. Here, gated decoupling measurement was applied in order to eliminate the nuclear Overhauser enhancement factor, and also the spectra were obtained with multiple scans at a pulse repetition time of 20s, considering the spin–lattice relaxation times (T_1) of 1.2s ($C_{(1)}$ in *trans*-isoprene unit) and 0.6s ($C_{(1)}$ in *cis*-isoprene unit). The observed values are in good agreement with those expected for the alignment of the ω-terminal isoprene residue, two *trans*-isoprene residues and 14–16 *cis*-isoprene residues aligned in that order. From these findings and the content of *trans*-isoprene residues in other polyprenols, it can be concluded that the polyprenols-15–20 have the structure (III).

Seasonal variation

The seasonal variation of the content of total lipids, polyprenyl acetates and polyprenols extracted from *Ginkgo biloba* leaves is shown in Fig. 5. Whereas the amount of total lipids was nearly constant throughout the season, a remarkable increase was observed for the content of polyprenyl

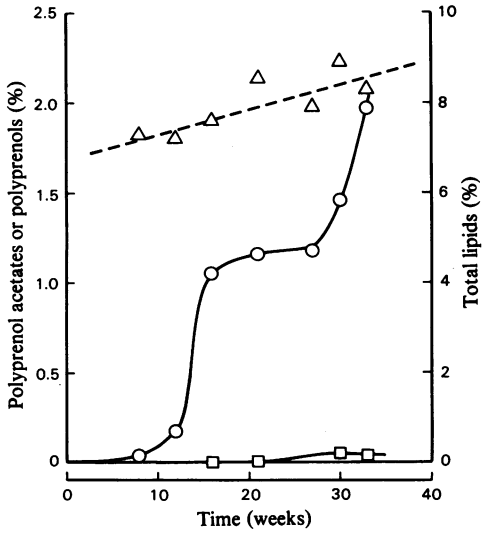


Fig. 5. *Seasonal variation of concentrations* (% *of dry wt. of leaves*) *of total lipids* (△), *polyprenyl acetates* (○) *and polyprenols* (□) *with the age of Ginkgo biloba leaves*
For experimental details see the text.

acetates with the maturing of the leaves. Similar observations have been reported for *Aesculus hippocastanum* and several other higher plants (Wellburn & Hemming, 1966*a*). The curve for polyprenyl acetates demonstrates the presence of two critical points, the first in early summer and the second in late autumn. The ultimate content of 2.0% of dry wt. of leaves was observed in late autumn. On the other hand, the distribution of chain length was essentially the same average length of isoprene residues of 17.7 ± 0.3 from the budding to leaf shedding, as in the case of castaprenols (Wellburn & Hemming, 1966*a*). The constancy of the distribution pattern was also observed for several other samples from *Ginkgo biloba* having different age, sex and climate where the tree has grown.

The synthesis of 'synthetic' dolichol was attempted by using the polyprenyl acetates as the starting material, and the 'synthetic' dolichol showed the same distribution of chain length as that extracted from pig liver (S. Suzuki, F. Mori, T. Takigawa, K. Ibata, Y. Ninagawa, T. Nishida, M. Mizuno & Y. Tanaka, unpublished work).

References

Brett, C. T. & Leloir, L. F. (1977) *Biochem. J.* **161**, 93–101

Burgos, J., Hemming, F. W., Pennock, J. F. & Morton, R. A. (1963) *Biochem. J.* **88**, 470–482

Dallner, G. & Hemming, F. W. (1981) in *Mitochondria and Microsomes* (Lee, C. P., Schalty, G. & Dallner, G., eds), pp. 655–681, Addison–Wesley Publishing Co., Reading

Doddrell, D. M. & Pegg, D. T. (1980) *J. Am. Chem. Soc.* **102**, 6388–6390

Feeney, J. & Hemming, F. W. (1967) *Anal. Chem.* **20**, 1–15

Gough, D. P., Kirby, A. L., Richards, J. B. & Hemming, F. W. (1970) *Biochem. J.* **118**, 167–170

Hemming, F. W. (1977) *Biochem. Soc. Trans.* **5**, 1223–1231

Jung, P. & Tanner, W. (1973) *Eur. J. Biochem.* **37**, 1–6

Richards, J. B. & Hemming, F. W. (1972) *Biochem. J.* **128**, 1345–1352

Rupar, C. A. & Carroll, K. K. (1978) *Lipids* **13**, 291–293

Stone, K. J., Wellburn, A. R., Hemming, F. W. & Pennock, J. F. (1967) *Biochem. J.* **102**, 325–330

Tanaka, Y. & Takagi, M. (1979) *Biochem. J.* **183**, 163–165

Tanaka, Y., Takeda, J. & Noguchi, K. (1982*a*) *U.S. Patent* 4338404

Tanaka, Y., Sato, H. & Kageyu, A. (1982*b*) *Polymer* **23**, 1087–1090

Tanaka, Y., Sato, H. & Kageyu, A. (1983) *Rubber Chem. Technol.* **56**, in the press

Waechter, C. J. & Lennarz, W. J. (1976) *Annu. Rev. Biochem.* **45**, 95–112

Wellburn, A. R. & Hemming, F. W. (1966*a*) *Phytochemistry* **5**, 969–975

Wellburn, A. R. & Hemming, F. W. (1966*b*) *Nature (London)* **212**, 1364–1366

Wellburn, A. R., Stevenson, J., Hemming, F. W. & Morton, R. A. (1967) *Biochem. J.* **102**, 313–324